

Optogenetics in primates: a shining future?

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To understand the functional role of specific neurons in micro- and macro-brain circuitry, health, and disease, it is critical to control their activity precisely. This ambitious goal was first achieved by optogenetics, allowing researchers to increase or decrease neural activity artificially with high temporal and spatial precision. In contrast to the revolution optogenetics engendered in invertebrate and rodent research, only a few studies have reported optogenetic-induced neuronal and behavioral effects in primates. Such studies are nonetheless critical before optogenetics can be applied in a clinical setting. Here, we review the state-of-the-art tools for performing optogenetics in mammals, emphasizing recent neuronal and behavioral results obtained in nonhuman primates.

Optogenetics in neuroscience

The brain fundamentally comprises glia and a variety of excitatory and inhibitory neurons. To understand the contributions of these cell types to normal and abnormal brain function [1], one must clarify the causal relation between the activities of these cell types and behavior. This ambitious goal can be achieved only by artificially altering cell activity while simultaneously measuring activity in the remainder of the circuitry and observing changes in behavior, preferably with physiologically relevant temporal resolution and in a reversible manner. Optogenetics is a recently developed method that enables researchers to control cell type-specific activity on a millisecond time-scale. By incorporating light-sensitive ion-conductance regulators (proteins called ‘opsins’, which are essentially ion channels and pumps) into neuronal membranes, these regulators can be activated by illumination with specific wavelengths. Depending on the type of opsin incorporated, neurons will be depolarized (activated) or hyperpolarized (deactivated).

In this review, we summarize the various aspects of optogenetics that are relevant to mammalian studies, particularly monkey research. We discuss important aspects of viral vectors, promoters, opsins, reporter genes, light delivery tools, and functional readout methods (Figure 1). Wherever possible, we refer to existing

review articles for in-depth discussions of the respective subdomains.

Viral vector construct

Viruses are ideal vehicles for delivering genes into cells, including neurons, and optogenetics relies on slightly transformed viruses to deliver the genetic material encoding opsins into neurons. Therefore, we first discuss the general properties of such vectors and how they are applied in optogenetics. A viral vector construct comprises: (i) a viral expression system used to deliver the opsin gene; (ii) a promoter fragment supporting cell type-specific targeting; (iii) the genetic information of the opsins; and optionally (iv) a reporter gene to visualize opsin expression levels and to determine cell-type specificity.

Viral vectors

The most commonly used viral vectors for gene transfer are developed from retro-, lenti- (LVV), adeno-, herpes simplex (HSV), and adeno-associated (AAV) viruses. Each has its characteristic cell infectivity and transgene capacity. LVV and AAV are the most widely applied vectors in optogenetics and have been successfully used to deliver opsins into mouse, rat, and primate brains (e.g., [2–6]). A general drawback of viral vectors is their limited packaging capacity and only genes that are relatively small, specific, and effective can be used. LVV can package larger gene fragments (approximately 8 kb [7]) compared with AAV systems (approximately 4 kb [8]). AAV can be more effective than LVV, because they are less temperature sensitive, smaller, and thus more likely to spread further away from the injection site. Moreover, higher titers of AAV [approximately 10^{12} genome copies (gc) per ml] can be obtained

Glossary

Cannula: tube that can be inserted into the body or brain, often for the delivery or removal of fluid and/or electrodes or for the gathering of data.

Gamma-band synchronization: synchronization of rhythmic neural activity across neural groups with a frequency of 25–100 Hz (typically 40 Hz).

Go-cue: change in the visual field or environment that serves as the signal to proceed to the next step of the task.

Phosphenes: phenomenon characterized by the experience of seeing light without retinal stimulation.

Photocycle: sequence of structural changes that a molecule (opsin) undergoes in reaction to light.

Receptive field: region of the receptor surface (e.g. retina) that must be stimulated to alter the firing of a downstream neuron.

Retinal: form of vitamin A that binds to opsins and is critical for animal vision.

Saccade: fast movement of an eye.

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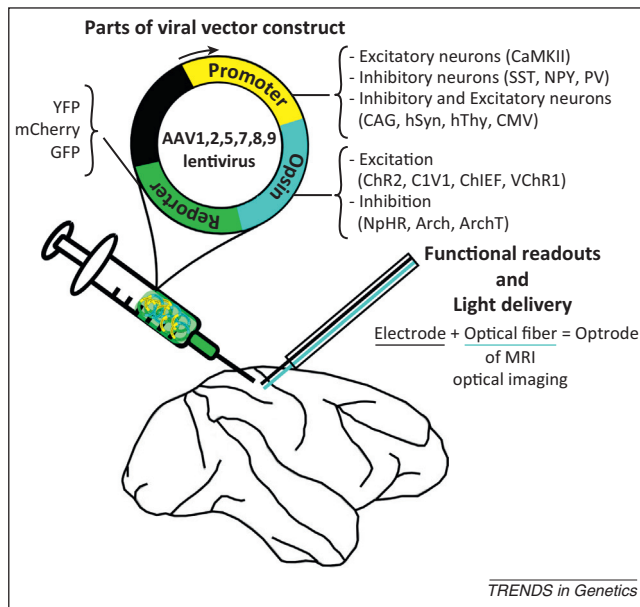


Figure 1. Variable parameters for conducting an optogenetic experiment. This schematic of part of a viral vector construct shows four variable components: (i) viral vector; (ii) promoter gene; (iii) opsin gene; and (iv) reporter gene. Examples are given of each component. The functional readout method and amount and/or frequency of light delivery can also be adapted. The black part of the viral vector map refers to other genes that are important for opsin expression in targeted neurons, but which are not discussed further.

compared with LVV (approximately 10^9 gc per ml), resulting in larger transduced tissue volumes. Therefore, LVV have been used to target subfields of brain structures, such as the subpart CA1 of the mouse hippocampus [9]. The expression pattern of various AAV serotypes in mammalian neurons is discussed in Box 1. In primates, optically induced modulation of neural activity was first observed after transduction using LVV [10]. In 2011, however, a second optogenetic study showed that AAV5 also safely and effectively delivered opsins into the monkey brain [11]. However, a recent monkey study [12] revealed positive functional and behavioral results after transduction with AAV but not LVV, which may indicate that AAV is more effective in primates than LVV.

Cell-type specificity

Cell-type specificity can arise from either the tropism of the viral vector itself or from promoter fragments in the vector

Box 1. AAV serotypes in mammalian neurons

AAV serotype 2 (AAV2) was the first AAV serotype cloned into bacterial plasmids and is the best-characterized natural serotype [90]. The most widely used AAV vectors are recombinant AAV2 (rAAV2) pseudotypes with various serotype-packaging systems (e.g., rAAV2/2 or rAAV2/5, referred to as AAV2 or AAV5, respectively). AAV5 disperses better in neural tissue than does AAV2 [91]. In mouse neurons, AAV serotypes 1, 2, 5, 8, 9, and hybrid serotype 2/7 successfully expressed opsins (e.g., [92–94]), whereas serotype 5 and a hybrid 5/10 transduced large volumes of neurons in rats (e.g., [24,95]). A monkey study compared different AAV serotypes (AAV1–6) in the substantia nigra and striatum [96,97]. Although AAV1 can transduce neurons and glia in striate cortex for two-photon imaging [98], AAV5 was the most efficient vector for both neurons and glia in monkeys [96,97].

construct that drive transgene expression. A comparison in mouse somatosensory cortex of AAV and LVV carrying the same promoter and transgene revealed that LVV transduced mainly excitatory neurons, whereas low-titer AAV2 vectors were expressed more in inhibitory neurons [13]. Other than cell type-specific promoters, one can use promoters that drive robust yet cell type-nonspecific expression in neurons [e.g., human elongation factor 1 α (EF1 α), cytomegalovirus (CMV), or CMV early enhancer/chicken beta actin (CAG)] [12,14–16]. Finally, depending on the viral vector, ubiquitously expressed promoters will also be expressed in glia.

Few cell type-specific promoters are sufficiently small to package in an AAV or LVV with the opsin and reporter gene. Glial fibrillary acidic protein (GFAP) promoter fragments applied in optogenetic experiments specifically drove transgene expression in astrocytes with LVV [5] and AAV [17]. Moreover, human synapsin I (hSyn1) [11,13,18] and Thy1 [11] promoters selectively target neurons (excluding glia) in rodents and primates. In addition, several inhibitory neuron-specific promoters, such as somatostatin (SST), calretinin (CR) and parvalbumin (PV), have been characterized [19], although none proved specific for subsets of inhibitory neurons. To the best of our knowledge, none of these inhibitory neuron-specific promoters have been used successfully in primates. By contrast, the calmodulin kinase II α (CaMKII α) promoter has been successfully applied for controlling only excitatory neurons in rodent as well as primate cortex (e.g., [3,10,20–24]). Finally, virus-compatible promoters for hypocretin, serotonin, and SST neurons have also been described [4,25–28].

Crucially, promoter specificity can differ across brain regions and species. Hence, each promoter should be histologically characterized for cell-type specificity within the context of the chosen viral vector, organism, and brain region.

Opsins: light-sensitive ion channels or pumps

Natural opsins. Many naturally occurring opsins have been discovered and applied in optogenetics. Microbial type I opsins require retinal (see Glossary) for photon absorption. When retinal is bound, functional opsin proteins are referred to as rhodopsins. When expressed in mammalian neurons, these can reliably control action potentials with millisecond precision [29]. Mammalian type II opsins are light-activated rhodopsins that initiate a G protein-coupled enzyme cascade (Box 2).

The first type I opsin identified was the haloarchaeal proton pump, bacteriorhodopsin (BR) [30,31]. A second class of microbial opsin genes encodes halorhodopsins (HR) [32], and a third encodes channelrhodopsins (ChR2) [33]. Blue light (approximately 470 nm) activates ChR2, initiating inward cation currents and depolarization of neurons. Excellent overviews of the microbial opsin family can be found in [9,34].

Bioengineered opsins. Bioengineering of existing opsin genes from different microorganisms by point mutations, codon optimization, or modifications affecting membrane trafficking has created a variety of chimeric rhodopsins

Box 2. Modulation of biochemical signaling

Microbial (type I) opsin genes described in the main text control neuronal excitability by directly manipulating membrane potential, whereas mammalian opsins (type II), are light-activated rhodopsins initiating a G protein-coupled enzyme cascade. Thus, optogenetic initiation of distinct biochemical signaling events can be achieved by constructing chimeras [99] of vertebrate rhodopsin and conventional ligand-gated GPCRs. In optogenetic literature, type II fusion proteins are referred to as optoXRs, allowing receptor-mediated intracellular signaling with temporal resolutions suitable for behavioral modulation in freely moving animals [36,100]. This ability to control biochemical signaling represents an exciting and rapidly growing aspect of optogenetics. Several groups have described optical control of small GTPases in cultured cells [101–103] using optically modulated protein–protein interactions.

with modified trafficking, kinetics, and light sensitivity (e.g., [21,35–41]; for an overview see Figure 2 and [42]). The first modification substituted algal for mammalian codons, resulting in greater expression [3,4,15,20], which is now standard practice for all newly engineered opsins. Mutations can be beneficial for one aspect (e.g., higher expression) but impact other ion-conductance properties (e.g., slower kinetics or stronger photocurrents). This can be circumvented by modifying ChR2 with a chimera approach [38,43], giving both stronger photocurrents and faster kinetics.

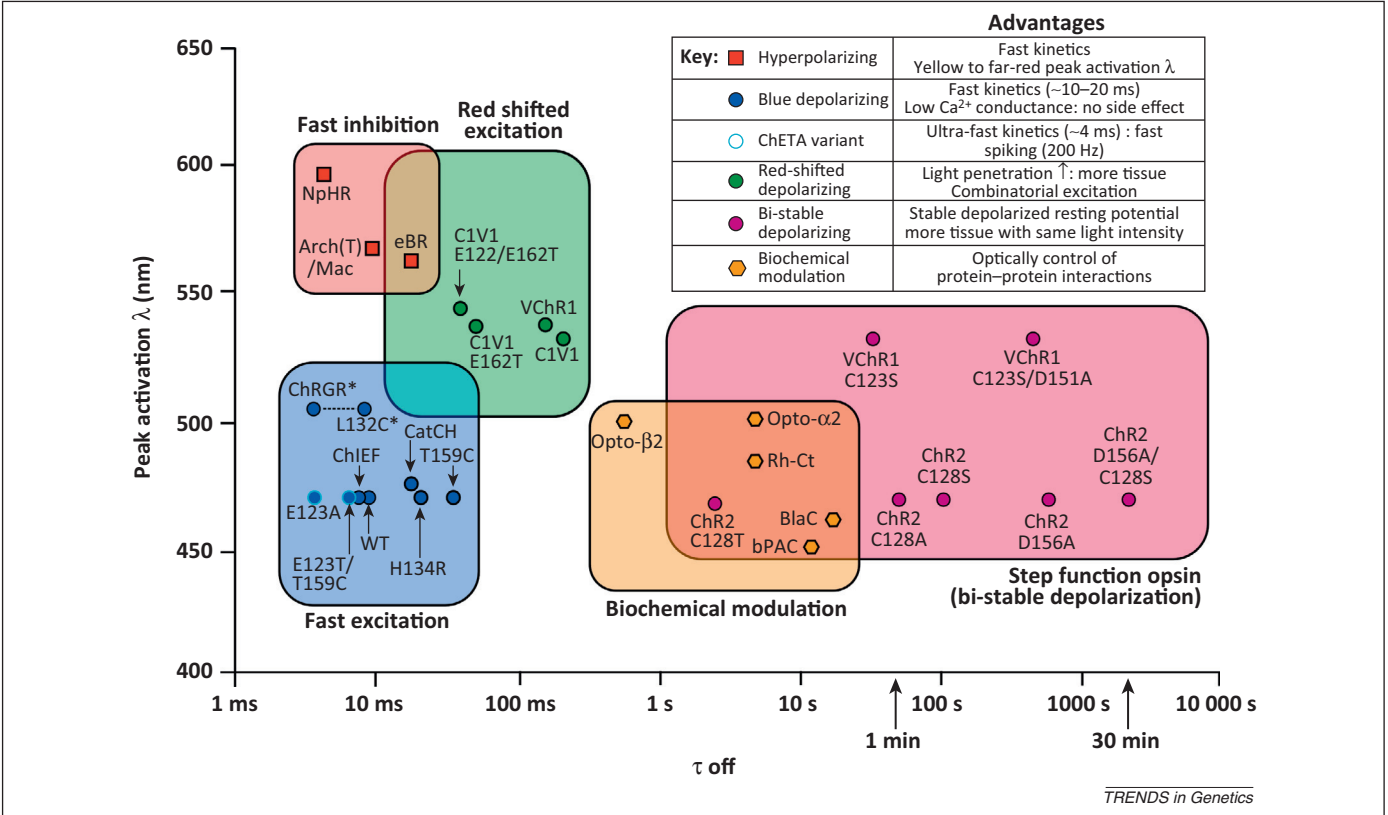
Optogenetic excitation

Red-shifted opsins. To mitigate heating, improve light penetration in brain tissue, and allow combinatorial

excitation *in vivo* [44], red-shifted ChR were developed. In 2008, *Volvox* ChR (VChR), a ChR excited at 590 nm, was created [45]. Further improvements of VChR1 culminated in the C1V1 family of ChRs, showing stronger photocurrents and red-shifted photosensitivity (peaks at approximately 560 nm), and allowing combinatorial excitation (see below) [44].

Step-function opsins. Step-function opsins (SFOs) represent another class of ChR2 mutants enabling bistable, step-like control of membrane potentials by having stable blue light-triggered photocurrents (conductance time up to 30 min [37,44]). Hence, one can achieve longer-lasting activations in targeted neurons by a short light pulse. Moreover, SFOs increase effective light sensitivity, yielding larger tissue volumes that can be stimulated at a given light intensity [37]. SFOs were successfully expressed in *Caenorhabditis elegans* [46] and primate neurons, allowing bidirectional control of cells [11].

Opsins with fast kinetics. Some experimental paradigms, in which one aims to mimic high natural firing rates of neurons, require fast kinetics for reliable spike rates at >40-Hz light stimulation. Substituting a glutamate residue in ChR2 for threonine (T) or alanine (A) accelerated channel closure from approximately 10 ms to approximately 4 ms. This acceleration significantly increased the fidelity of fast optogenetic control [40]. Opsins of this class are termed ChETAs [substituting a glutamate residue (E) in ChR2 for threonine (T) or alanine (A)], indicating the T/A



mutation. The major caveat of these opsins is their reduced effective light sensitivity for long light pulses, because fewer channels remain in the open (conducting) state.

Two other mutants, ChEF (ChR2 chimera with a cross-over site at loop E-F) and ChIEF [a variant of ChEF with isoleucine (I) mutated to valine], display the most consistent photocurrent responses with continuous or high-frequency, pulsed light stimulation [38]. ChIEF is faster than ChEF and comparable to ChR2, but has slightly reduced light sensitivity relative to ChR2 [47,48].

Another ChR2 variant, calcium-translocating ChR (CatCh) [41], contains enhanced Ca^{2+} permeability, giving accelerated response times and an approximately 70-fold increase in light sensitivity over ChR2. The enhanced light sensitivity and fast kinetics originate from the relatively high light-gated Ca^{2+} influx, which elevates the inner membrane surface potential and activates Ca^{2+} -activated large conductance potassium (BK) channels. Thus, the response time depends on the presence of these BK channels in the expressing neurons [41].

Optogenetic inhibition

In parallel with engineered light-sensitive proteins, opsins from many species were screened for inhibitory properties. Expression of HR causes hyperpolarization and inhibition. In addition to the light-sensitive chloride pump HR [49], light-driven proton pumps Arch and ArchT cause neuronal silencing by pumping intracellular protons into extracellular medium [21,50,51]. In macaque parietal neurons, expression of this silencer resulted in decreased spike rates when transduced neurons were illuminated with yellow (575 nm) light [21]. HR from *Natronomonas* (NpHR) requires constant light of approximately 590 nm to complete its photocycle and is further optimized, improving expression levels and light sensitivity. Trafficking problems were mitigated by specific gene modifications [35,52] to improve membrane localization and to allow stronger currents in primates (eNpHR2.0; [11,53]). The next version, eNpHR3.0 exhibited enhanced photocurrents driving inhibition with yellow- or far red-shifted wavelengths (up to 680 nm; [54]), and demonstrated behavioral effects of optogenetic inactivation in freely moving animals [55,56].

The photocurrent of these hyperpolarizing opsins peaks at or above approximately 590 nm. At these wavelengths, ChR2 (peak approximately 470 nm) shows no or minimal response. These properties allow exciting experiments in which neural activity is controlled bidirectionally. For example, one could increase the neural activity of excitatory cells within a particular brain region with ChR2 behind a CaMKII α promoter. Hypothetically, to decrease neural activity of inhibitory cell types within the same region, the eNpHR3.0 opsin can be expressed under control of an SST promoter. As mentioned above, the SST promoter has not yet been used in primates. Thus, bidirectional optogenetic control in primates will only be feasible when new cell type-specific promoters are developed or larger viral vectors can be used for gene delivery.

Reporter genes

The final gene fragment to be discussed that has been incorporated into viral vector constructs for optogenetics is

the reporter gene. Fluorescent proteins (FPs) [57] are widely used in optogenetics to quantify opsin expression and assess vector transduction efficiency and cell type-specificity. Most viral vector systems contain GFP [58], which is excited by blue light (approximately 490 nm) and emits green light (approximately 510 nm). High green background fluorescence makes red FP mCherry (excitation and emission peaks approximately 560–590 and 580–610 nm, respectively) or yellow FP (excitation and emission peaks at 514 and 527 nm, respectively) attractive alternatives for use in optogenetic viral vector systems (e.g., [1,22]).

Besides opsin expression levels, multicolor labeling with cell type-specific antibodies can be used to assess opsin cell-type specificity [59].

Functional readouts

After injecting viral vector constructs into the target region, one can monitor the effect of optical stimulation on neural activity either by local electrophysiological measurements, or (more) globally but indirectly by functional MRI (fMRI) or optical imaging.

In 2007, *in vivo* electrophysiology of optogenetic-induced neural modulation was first performed by gluing an electrode to an optical fiber, creating the 'optrode' [1]. Subsequently, more sophisticated optrodes were developed, such as those used to collect electrophysiological signals from multiple sites in freely moving animals [60,61] (Box 3).

Opto-fMRI

Combining optogenetics with fMRI permits the investigation of the functional impact of optogenetic perturbations on large-scale brain networks. In 2010, the first rat opto-fMRI (ofMRI) experiment was performed [24]. The results showed that direct excitation of principal neurons elicited positive hemodynamic responses and that optical stimulation of remote axons can produce fMRI signal changes. Subsequent rat ofMRI studies [6,62,63] have shown the importance and usefulness of ofMRI in connectivity and plasticity studies (reviewed in [64]).

Recently, ofMRI in monkeys has revealed functional network changes induced by optically stimulating frontal neurons [12]. Optogenetic-induced network changes showed remarkable similarities with published anatomical

Box 3. Optrodes

In 2010, an optrode was attached to a 16-site silicon probe for recording in anesthetized animals [104], followed by a similar silicon probe for chronic awake recordings [61]. The 'optetrode' comprised 16 microwires (which are cheaper and more robust than silicon) wound into tetrode bundles. Such optetrodes allowed colocalized multi-tetrode electrophysiological recording and optical stimulation in freely moving mice [105]. Shortly thereafter, 'optrode-microelectrode arrays', or 'optrode-MEAs', emerged. An optical fiber was integrated into an intracortical 2D or 3D microelectrode array [106,107]. These optrode-MEAs enabled optical stimulation of multiple sites simultaneously while recording from all microelectrodes.

An elegant approach for determining transduction efficiency *in vivo* was developed in 2012 [108]. These researchers constructed glass-coated tungsten microelectrodes encasing optical fibers for optogenetic stimulation and measuring ChR2-EYFP expression in deep brain structures of nonhuman primates.

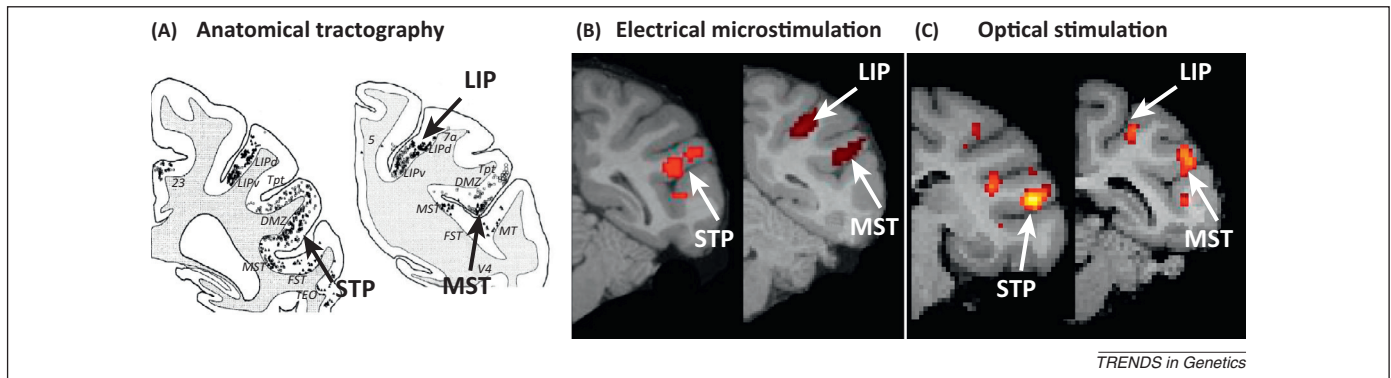


Figure 3. Comparison between (A) anatomical tractography, (B) electrical microstimulation (EM), and (C) optical stimulation. Injections of tracer in the frontal eye fields (FEF) of macaque monkey resulted in labeled cells in the lateral intraparietal area (LIP), the medial superior temporal area (MST), and the superior temporal polysensory area (STP) [65,66]. Functional MRI (fMRI) combined with EM of the FEF resulted in fMRI activations in LIP, MST, and STP [67]. Monkey opto-fMRI with ChR2-transduced neurons in FEF also showed an increase in fMRI signal in LIP, MST, and STP [12]. Reprinted, with permission, from [12] (C), [66] (A), and [67] (B).

tractography and electrical microstimulation (EM) data from the same brain regions (Figure 3) [65–67], despite the fact that the behavioral effects induced by the two methods were different (see below).

Optogenetic-induced changes in behavior

Most optogenetic studies have been performed in invertebrates or rodents and report several intriguing behavioral effects. An early optogenetic application using freely moving mice showed that light activation of transduced motor cortex readily induced extensive motor activity [3]. It has since been demonstrated that complex changes in state or behavior can be induced by optical modulation of critical neurons participating in those processes, including habit formation [68], sleep [4,69], reward and addiction [70–73], fear and anxiety [56,74], and depression [75]. Despite remarkable results in rodents, only recently have optogenetic-induced changes in behavior in primates been reported. Given that primate experiments typically last much longer than rodent studies, safety aspects of optogenetics become an important issue (Box 4).

Optogenetics in primates

Neural effects

The first optogenetic study of nonhuman primates was performed in 2009 [10]. Months after viral transduction, histology revealed widespread ChR2-GFP expression in healthy-looking frontal eye field (FEF) neurons. These opsins, under CaMKII-promoter control, were delivered to FEF neurons using LVV and were preferentially expressed in excitatory neurons. Single-cell recordings showed temporally precise optical activation, presumably of ChR2-expressing cortical excitatory neurons. Surprisingly, optical stimulation of FEF neurons evoked no eye movements as typically observed following EM of the same region. Besides the expected increase in single-cell activity, widespread neuronal suppression was observed. This important result indicates that neural downstream effects within the local microcircuitry are elicited. Most likely this is caused by the recruitment of inhibitory neurons downstream of the (transduced) excitatory neurons directly stimulated by light. These excited interneurons, in turn, suppress other neurons within the local microcircuitry. Indeed, in contrast to the short latencies (approximately 9 ms) of excited units after

stimulation onset, suppressed units began decreasing their firing rates significantly later (approximately 31 ms), which indicates a polysynaptic effect. Hence, one always must consider the effect of cell type-specific optogenetic stimulation on the entire local microcircuitry to interpret its global functional and behavioral effects [76]. Bidirectional optogenetics, during which particular cell types (e.g., particular inhibitory neuron types) are silenced, will be critical to isolate the function of (other) light-activated neurons.

Two years later, AAV5, with a synapsin and a Thy-1 promoter to express ChR2, ChR2-C128S, and eNPHR2.0, was injected into monkey (pre)motor cortex to investigate transduction efficiencies [11]. These constructs induced strong expression in cortical neurons, and transduced neurons responded well to light modulation. Unexpectedly,

Box 4. Safety concerns

A major concern of optogenetics is toxicity due to opsin overexpression. Given that rodent studies typically last only a few months, long-term effects on neurons transduced with opsins are not fully understood. Months after transducing monkey brain using LVV, researchers observed widespread expression of ChR2-GFP without obvious histological abnormalities in neurons or glia, or immune reactions at cellular or antibody levels [10]. By contrast, another research group found ChR2-eYFP aggregations linked to overexpression that could affect cell health [11]. These aggregations can be mitigated using less potent promoters (Thy-1 instead of hSyn), LVV instead of AAV5 (or a lower virus titer), or enhanced trafficking. Given that these studies lasted less than 1 year, long-term effects of opsins in primates should be thoroughly understood before considering translational applications.

Other issues concern the optrodes and phototoxicity. Optical fibers are typically approximately 200 μm in diameter. Repeated use of such fibers obviously damages tissue. Behavioral and fMRI results of a recent nonhuman primate optogenetic study were transient, attributable to tissue damage caused by optical fibers [12]. To mitigate this problem, one could use chronically implanted optical fibers or arrays, apply red-shifted opsins [44,45,54], and when targeting superficial structures, replace the dura with transparent silicone film. For applications in mice, red-shifted opsins can be used to stimulate optically deep neurons noninvasively. However, red light will not penetrate sufficiently to reach deep brain structures in monkeys. Red-shifted opsins will also reduce the amount of light necessary to (in)activate transduced neurons, thus reducing the risk of phototoxicity. Phototoxicity is a phenomenon known from *in vivo* cell imaging, where illumination selectively kills cells expressing FPs [109]. However, when using low laser intensities, phototoxicity should not be a major safety concern.

regardless of promoters, no opsin expression appeared in superficial cortical layers and again, unlike EM and despite strong effects on neuronal activity, optical stimulation of cortical motor and premotor neurons evoked no measurable behavioral effects.

Meanwhile, primate neurons were also transduced with the light-sensitive neuronal silencer, ArchT [21]. As discussed above, this modified Arch supports light-driven attenuation of neural activity with high efficacy and fast kinetics. ArchT triples the light sensitivity of Arch, doubling the affected volume of tissue with the same irradiance, which can be important for behavioral optogenetic studies in primates. Concurrently, AAV5-ChR2 and AAV5-NpHR were injected into primate lateral intraparietal area (LIP) and neuronal activity was recorded in that same position using a single coaxial optrode inside specially designed cannulas [77].

Behavioral effects

So far, only three studies have reported optogenetic-induced behavioral effects in nonhuman primates [12,18,78]. In the first study, two monkeys were trained on a visually guided saccade task [12] (Figure 4A). After a fixation period, four white distracters and one green target appeared. After a pause, one white distracter decreased in luminance (go-cue), allowing the monkey to make a saccade to the target. Frontal regions showing task-related fMRI activity were injected with AAV5-CAG-ChR2-eGFP using neuronavigation. Comparison of optically stimulated with nonstimulated task trials revealed a consistent and significant interaction between reaction time (RT) and target location: stimulation resulted in faster saccadic RTs by approximately 10%. Strikingly, the saccade metrics were not affected by optogenetic stimulation, as typically observed after EM.

The second monkey study described optogenetic-induced saccades towards the receptive field (RF) of ChR2-transduced neurons in primary visual cortex (V1 [18];

Figure 4B). After injecting rAAV1-Syn1-ChR2(H134R)-mCherry into monkey V1, blue-light stimulation was administered during a fixation trial. After the fixation period, the fixation dot disappeared and the monkey made spontaneous saccades. When blue light was administered at the transduced sites immediately after dot disappearance, spontaneous saccades concentrated around the RFs of the stimulated neurons. This indicates that, most likely, phosphenes are produced by optogenetic stimulation of V1.

Finally, in the most recent study, intermediate-layer neurons in superior colliculus (SC) that discharge before and during saccades were transduced using an AAV8-CAG-ArchT-GFP vector [78]. Green illumination activated the light-driven proton-pump ArchT, eliciting saccadic deficits and reducing neuronal activity at the stimulation site. The shift in saccade endpoint depended upon the proximity of the SC neurons underlying the saccade to both the injection and optrode sites. Neurons closer to either site exhibited stronger behavioral effects. Moreover, saccade endpoints shifted primarily away from the injection site rather than towards the illumination site.

A profound discrepancy between optical and electrical stimulation has been reported in monkey optogenetic studies [10–12]. In the second study, the authors hypothesized that the lack of optogenetic-induced hand and/or arm movements is due to a too-small volume targeted by the optrode compared with the volume of neurons typically activated with EM [11]. In another study, two fibers were inserted simultaneously to overcome this challenge, but still no saccades were evoked by optical stimulation; only saccade latencies were affected [12]. This suggests that either the stimulated tissue volume was indeed too small or that different neural populations were affected by optogenetics. The latter hypothesis can be explained by layer-specific viral vector transduction efficiency [11] and by a different, distributed, and sparse pattern of activated neurons through axonal activation after EM [79]. Combining

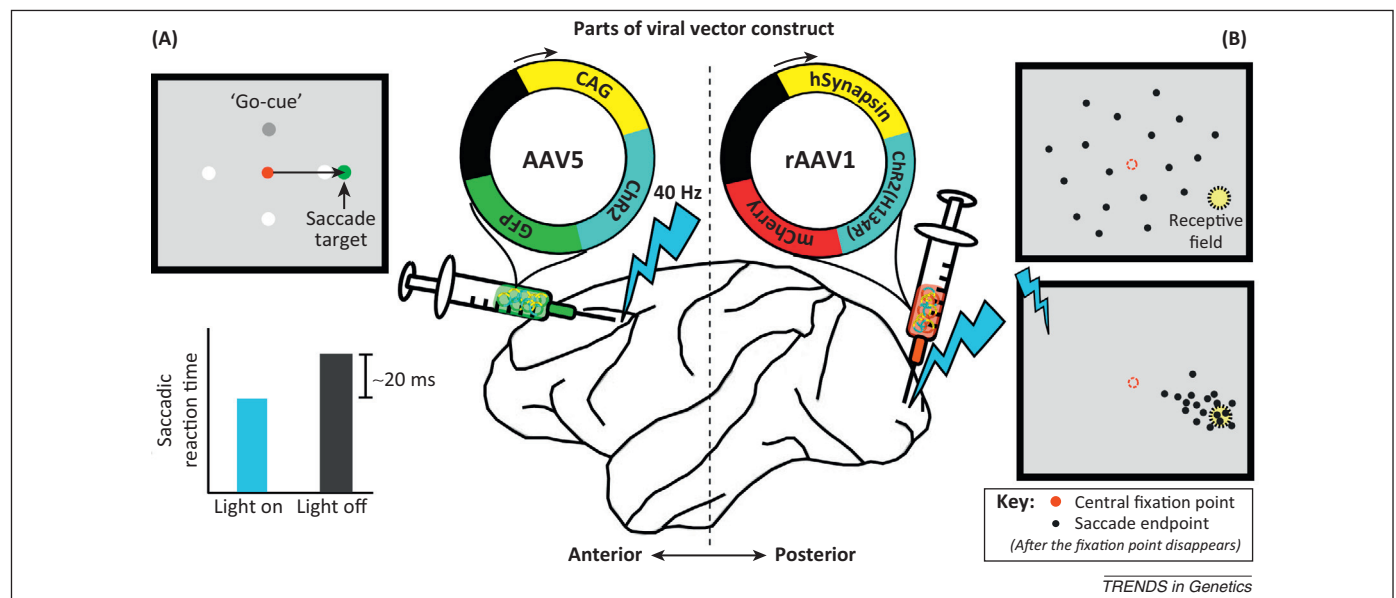


Figure 4. Behavioral effect of optical stimulation in nonhuman primates. In the study in (A) [12], frontal neurons were transduced with an AAV5-CAG-ChR2-GFP vector, and optical stimulation of these neurons decreased the saccadic reaction time during a visually guided saccade task. In the study in (B) [18], neurons in the primary visual cortex were transduced with rAAV1-hSyn1-ChR2-mCherry and optical stimulation resulted in spontaneous saccades towards the receptive fields of these neurons. Reprinted, with permission, from [12] (A) and [18] (B).

optical stimulation with two-photon calcium imaging as in the latter EM study is essential to reveal differences between local optogenetic and EM activated microcircuits. A possible remedy for increasing the number of neurons affected by optical stimulation may be the use of red-shifted opsins, because red light spreads further in brain tissue. Another typical difference between optical and EM is the frequency of stimulation. In optogenetic primate studies, the frequency of optical stimulation ranged between 10 Hz and 50 Hz, whereas EM frequencies typically range between 100 Hz and 350 Hz [80]. Hence, a parametric study comparing stimulation frequencies across methods (e.g., using opsins with fast kinetics, such as ChETA or ChIEF [48]) is needed to clarify frequency-dependent functional differences between optogenetic stimulation and EM.

Concluding remarks

Genetic targeting of specific cell types has proved very successful in transgenic mice, facilitated by the Cre-lox system [42]. Regrettably, for the foreseeable future, monkey and other genetically intractable animal models must rely on viral construct injections to express opsins in neurons. Unfortunately, targeting specific cell types with viral technologies has been only moderately successful in monkeys. The number of cell type-specific promoters small enough to be packaged within existing viral vector systems is limited. Additionally, interactions between target cells and viral particles are poorly understood and cellular regulation mechanisms of virally mediated gene expression remain unpredictable. Therefore, the development of viral technologies targeting specific cell types in monkeys is an important challenge for future optogenetic studies.

For example, with the present optogenetic tools, it is difficult to target inhibitory cell types in monkeys. Nonetheless, specific manipulation of such cells could yield major breakthroughs in studies focusing on the relevance of gamma-band synchronization for cognition. For instance, inhibitory GABAergic basket cells impose rhythmically synchronized inhibition onto local excitatory neurons, causing them to fire rhythmically. It has been hypothesized that basket cells play an important role in gamma-band synchronization, creating long-range dynamic communication channels between brain regions [81]. Alternatively, it has been suggested that gamma-band synchronization simply reflects properties of a neural normalization mechanism in which the response of a neuron is normalized by the activity of many neighboring neurons [82]. Hence, precise optogenetic stimulation of inhibitory cells would allow researchers to show or refute causal links between their firing, gamma-band synchronization, and higher cognitive functions.

Another exciting challenge for primate optogenetics would be to target dopaminergic neurons, requiring the development of specific promoters for these cells. Optogenetic stimulation and/or inactivation of dopamine neurotransmission will be critical for understanding the neurobiological basis of dopaminergic-based behavior and disorders (e.g., Parkinson's disease and attention deficit disorders). Unlike EM, optogenetics would allow for stimulation of only the relevant neurons for the

disease, leaving other neighboring regions untouched. Therefore, this would provide a framework to manipulate effectively dopamine patterns in disease, thereby reducing typical adverse effects of current state-of-the-art clinical treatments.

Coexpression of red-shifted with blue light-activated opsins in different cell types within a particular brain region would permit the independent (de)activation of these different neuron populations. Achieving bidirectional control at a single-cell rather than a regional level would be one of the holy grails for investigating with unprecedented precision how specific neurons shape global brain activity and behavior. For example, cell type-specific bidirectional optogenetics would enable control of activity during different stages of trials when monkeys perform a cognitive task, thereby investigating the contribution of specific cell types to different mental operations engaged during trial execution. For these purposes, it is necessary to further develop and characterize the cell-type specificity of the viral vector delivery technology in primates. Achieving this ambitious goal will require an increase in the packaging capacity of viral vector systems to host different opsins in one construct [8], possibly by introducing novel viral vectors in the optogenetic field.

An exciting tool for remotely controlling neuronal activity without optics has recently been developed based on a class of designer G protein-coupled receptors (GPCRs) that selectively respond to small molecule ligands, allowing direct interference with G protein signal transduction pathways (reviewed in [83,84]). This new tool extends research from the 1990s in which the *Drosophila* allatostatin receptor (AlstR), a GPCR, was found to respond exclusively to allatostatin (AL) [85]. In 2002, this AlstR/AL receptor–ligand system was used to silence activity in cortical ferret neurons [86] and, in 2012, to induce retinotopic-specific deficits in a detection task after activating AlstR in macaque V1 [87].

The recently developed designer receptors exclusively activated by a designer drug (DREADDs) respond 5–15 min after systemic administration and the response lasts several hours. Given that DREADD ligands are systemically bioavailable, delivery is less invasive than inserting optical fibers. Moreover, such approaches mimic native *in vivo* signaling in awake, freely moving animals. Thus, minimally invasive DREADD-like approaches are promising, especially for translational applications where the temporal resolution of the perturbations may be less critical.

Recent reports of optogenetic-induced behavioral effects in primates bring us a step closer to introducing optogenetics in humans. With optogenetics, complex behaviors in primates can be studied to help understand and treat neuronal deficits underlying human neurological diseases [88]. Pioneering this exciting endeavor, some groups are already planning clinical trials to treat blindness and spinal cord injury with optogenetic techniques [53,89].

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